Carbon and gold electrodes as electrochemical transducers for DNA hybridisation sensors.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biosensors and Bioelectronics 19 (2004) 515–530
BIOSENSORS & BIOELECTRONICS
www.elsevier.com/locate/bios

Review

Carbon and gold electrodes as electrochemical transducers for DNA hybridisation sensors

Fausto Lucarelli [a,*], Giovanna Marrazza [a], Anthony P.F. Turner [b,1], Marco Mascini [a]

[a] *Dipartimento di Chimica, Universita' degli Studi di Firenze, via della Lastruccia 3, 50019 Sesto Fiorentino, Firenze, Italy*
[b] *Cranfield University, Silsoe, Bedfordshire MK45 4DT, UK*

Received 28 November 2002; received in revised form 24 June 2003; accepted 30 June 2003

Abstract

Genosensor technology relying on the use of carbon and gold electrodes is reviewed. The key steps of each analytical procedure, namely DNA-probe immobilisation, hybridisation, labelling and electrochemical investigation of the surface, are discussed in detail with separate sections devoted to label-free and newly emerging magnetic assays. Special emphasis has been given to protocols that have been used with real DNA samples.

Keywords: Carbon electrodes; Gold electrodes; DNA hybridisation; Electrochemical DNA biosensors; Genosensors; DNA chips; DNA sensors; PCR

1. Introduction

The field of molecular diagnostics has expanded rapidly in the past decade, influenced greatly by the progress of the Human Genome Project. As new genes are discovered and shown to be implicated in disease, primary patient diagnosis, carrier detection and prenatal diagnosis will continue to increase exponentially. To improve patient care, molecular diagnostic laboratories have been challenged to develop new tests that are reliable, cost-effective and accurate and to optimise existing protocols by making them faster and more economical. Analysis of gene sequences and the study of gene regulation play fundamental roles in the rapid development of molecular diagnostics and in drug discovery. Detection of infectious species and genetic mutation at the molecular level opens up the possibility of performing reliable diagnosis even before any symptoms of a disease appear. Additionally, the development of novel therapeutics based on the regulation of gene expression provides revolutionary new opportunities in the area of pharmaceutical science.

Molecular diagnostics based on the analysis of genomic sequences have offered a highly sensitive and quantitative method for the detection of infectious disease pathogens and genetic variation. Conventional methods for the analysis of specific gene sequences are based on either direct sequencing or DNA hybridisation. Because of its simplicity, DNA hybridisation is more commonly used in the diagnostic laboratory than the direct sequencing method. In DNA hybridisation, the target gene sequence is identified by a DNA probe that can form a double-stranded hybrid with its complementary nucleic acid with high efficiency and extremely high specificity in the presence of a mixture of many different, non-complementary, nucleic acids. DNA probes are single-stranded oligonucleotides, labelled with either radioactive or non-radioactive material, to provide detectable signals for DNA hybridisation. Radioactive labels are extremely sensitive, but have the obvious disadvantage of short shelf life, risks associated with exposure of personnel to radiation, cost, storage and disposal problems. On the other hand, non-radioactive probes, such as enzymatic or luminescence labels, are less sensitive and flexible in terms of design and application. Therefore, large-scale, routine clinic screening based on gene diagnostics is limited by the currently available technologies. Development of faster, simpler and label-free methods will significantly improve the applications of DNA molecular diagnostics.

* Corresponding author. Tel.: +44-1525-863005; fax: +44-1525-863360.
E-mail addresses: fausto.lucarelli@unifi.it (F. Lucarelli), a.p.turner@cranfield.ac.uk (A.P.F. Turner).
[1] Co-corresponding author.

0956-5663/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0956-5663(03)00256-2

Recent advances in automated DNA synthesis and the convenient site-specific labelling of synthetic oligonucleotides with suitable functional moieties, coupled with advances in microelectronics, have accelerated the development of biosensors for the analysis of specific gene sequences. A biosensor is a device that incorporates a biologically active layer as the recognition element and converts the physical parameters of the biological interaction into a measurable analytical signal. A DNA biosensor (or genosensor) employs an immobilised DNA as the recognition element. Electrochemical DNA biosensors rely on the conversion of the base-pair recognition event into a useful electrical signal. Electrochemical devices are highly sensitive, inexpensive, easy-to-use, portable and compatible with microfabrication technologies. Thus, they seem to be excellent candidates for the rapid and inexpensive diagnosis of genetic diseases and for the detection of pathogenic biological species of clinical interest. The detection of specific DNA sequences related to genetically modified organisms (GMO) is also an emerging monitoring task.

Typically, the design of an electrochemical genosensor involves the following steps:

(1) immobilisation of the DNA probe;
(2) hybridisation with the target sequence;
(3) labelling and electrochemical investigation of the surface.

Optimisation of each step is required to improve the overall performance of the devices. Recently, the use of magnetic beads has substantially changed the possible electrochemical hybridisation assay scenarios. The major problem of several biosensing formats (e.g. the non-specific adsorption of non-hybridised oligonucleotides onto the probe-modified electrode surface) has been overcome by conducting the hybridisation and the transduction steps at different surfaces (magnetic beads and unmodified electrodes, respectively). The efficient magnetic isolation of the duplex allowed the discrimination against non-hybridised DNA, including non-complementary sequences and an excess of mismatched oligonucleotides (Wang et al., 2001a; Palecek et al., 2002a). Nevertheless, in such assays the electrode surface acted merely as an electrochemical transducer and could not be considered as a true DNA biosensor.

Recent progresses in the development of electrochemical DNA-hybridisation biosensors have been summarised in some excellent reviews (Pividori et al., 2000; Yang et al., 1997; Wang, 2000; Palecek and Fojta, 2001; Palecek, 2002). Hence, this review will be focused on electrochemical DNA biosensors and sensors developed using carbon and gold electrodes as the transducer. Each step in the assays procedure will be discussed in detail, highlighting advantages and disadvantages of different methods. Special emphasis will be given to papers describing the analysis of real samples (e.g. PCR products).

2. Carbon electrodes

Several carbon-based surfaces have been investigated as electrochemical transducers for DNA hybridisation biosensors. Carbon paste (Wang et al., 1996a), pencil lead (Wang et al., 2000) and screen-printed electrodes (Marrazza et al., 2000) have been widely used by many groups to assemble DNA biosensors. Other electrodes, such as carbon fibre electrodes (Caruana and Heller, 1999), have been also successfully employed. Although glassy carbon electrodes are not suitable for direct detection of DNA oxidation peaks, (Cai et al., 1996) their utility for direct covalent attachment of DNA probes (Millan et al., 1992) or electropolymerisation of probe-supporting redox hydrogels (de Lumley-Woodyear et al., 1996) has been demonstrated.

To date, the use of disposable strips, that obviate the need for a regeneration step, seem to be the most promising approach, since it meets the needs of decentralised genetic testing.

2.1. Immobilisation of the DNA probe

DNA probes are typically short (18–40-mer) oligonucleotides that are able to hybridise with specific target sequences. While earlier work employed simple DNA probe sequences as a model (e.g. oligo $d(G)_{20}$), recent reports describe the use of disease- or microorganisms-related oligonucleotide sequences. Besides DNA probes, Wang et al. (1996b) has demonstrated that significantly enhanced selectivity can be achieved by the use of peptide nucleic acids (PNA) probes. Such DNA analogues, possess an uncharged pseudopeptide backbone (instead of the charged phosphate-sugar backbone of natural DNA). Because of their neutral backbone, PNA probes offer greater affinity in binding to complementary DNA, and improved distinction between closely-related sequences (including single-base mismatches). Such mismatch discrimination has a particular importance in the detection of disease-related mutations.

The probe immobilisation step plays the major role in determining the overall performance of an electrochemical DNA biosensor. The achievement of high sensitivity and selectivity requires maximisation of the hybridisation efficiency and minimisation of non-specific adsorption, respectively. Control of the surface chemistry and coverage is essential for assuring high reactivity, orientation, accessibility and stability of the surface-confined probe as well as for minimising non-specific adsorption events.

2.1.1. Adsorptive accumulation

Adsorption at controlled potential is the simplest method to immobilise DNA (or PNA) probes onto pretreated carbon-based surfaces (Wang et al., 1996a; Marrazza et al., 1999; Erdem et al., 1999). The method does not require special reagents or nucleic acid modifications. An oxidative pretreatment of carbon surfaces is necessary to enhance the adsorptive accumulation of DNA (Wang et al., 1995).

On the other hand, the potential applied during immobilisation (generally +0.5 V versus Ag/AgCl) enhances the stability of the probe through the electrostatic attraction between the positively charged surface and the negatively charged sugar-phosphate backbone of DNA (Wang et al., 1998a). The inherent oxidation signals of DNA bases onto carbon surfaces can be used to monitor the immobilisation process. However, using this immobilisation procedure, stringent washing steps (necessary to insure a high selectivity against mismatched sequences) cannot be performed, because of the desorption of the non-covalently bound hybrid. Moreover, because of the multiple sites of binding, most of the immobilised DNA probe is not accessible for hybridisation, resulting in poor hybridisation efficiency.

2.1.2. Bulk-modification of carbon paste

Wang et al. (1998b) reported the use of oligonucleotide bulk-modified carbon paste electrodes. The DNA probe was immobilised onto titanium oxide-coated silica beads (through the reaction between the titanium oxide with the phosphate backbone of the oligonucleotide) and then uniformly dispersed within a carbon paste matrix. The immobilised probe retained its hybridisation activity despite the confinement in the interior of the carbon paste; each measurement was performed on a new surface, obtained by a simple extrusion/polishing procedure.

2.1.3. Attachment of biotinylated probes to avidin-coated surfaces

Mascini's group reported the immobilisation of synthetic oligonucleotides onto disposable carbon strips using an avidin–biotin based procedure (Marrazza et al., 1999). This procedure involved the controlled formation of avidin layers (by adsorption) onto the electrode surface and the subsequent binding of the DNA probe biotinylated at the 5′-end. However, the avidin layer inhibited the electrochemical oxidation of the daunomycin, the indicator used to detect the hybridisation; a shorter and simpler immobilisation by adsorption at controlled potential allowed a better reproducibility and sensitivity.

2.1.4. Coupling of biotinylated probes to colloidal gold-neutravidin conjugated layers

An array, consisting of 10 screen-printed carbon electrodes, was described by Wojciechowski et al. (1999). Carbon electrodes were first modified with a colloidal gold-neutravidin conjugated layer. This platform allowed the development of different protocols for the electronic detection and quantification of nucleic acids, based on the use of biotinylated DNAs and a horseradish peroxidase (HRP) enzyme label.

2.1.5. Carbodiimide covalent binding to activated surfaces

Millan et al. (1992) reported the covalent immobilisation of synthetic oligonucleotides onto electrochemically oxidised glassy carbon surfaces by using a water-soluble carbodiimide. Experimental results suggested that covalent binding to the electrode surface occurred through guanine bases of DNA. The immobilisation process was monitored by using $Co(bpy)_3^{3+}$, a minor groove binder that was preconcentrated at the electrode surface because of its interaction with DNA. The immobilisation chemistry was later improved by employing *N*-hydroxysulfosuccinimide (NHS) with a water-soluble carbodiimide (EDC) reagent to activate carboxylate groups on the glassy carbon electrode surface (Millan and Mikkelsen, 1993). Single-stranded DNA was covalently bound to these groups through deoxyguanosine residues.

Carbon paste electrodes, modified by inclusion of 5% stearic acid, were also explored for covalent immobilisation of DNA (Millan et al., 1994). The selected DNA probe, enzymatically elongated at the 3′-end with dG residues, was immobilised at stearic acid-modified electrodes by using the water-soluble carbodiimide and NHS to activate the carboxylate groups on the surface. Such a covalent immobilisation procedure resulted in a high hybridisation efficiency of the immobilised DNA probes; moreover, the regeneration of the probe-modified surface was achieved by simple rinsing in hot water.

The covalent immobilisation of synthetic oligonucleotides having a C6 spacer and a terminal amino group onto conductive polycarbonate/carbon fibre electrodes was explored by Schülein et al. (2002). The immobilisation proceeded through the formation of amide bonds between the carboxylic functionality at the electrode surface and the amino-terminal end of the oligonucleotides. The electrode coverage was estimated using fluorescein-labelled oligonucleotides.

2.1.6. Attachment to polymer-coated surfaces

Heller's group reported the immobilisation of short oligonucleotides onto a film of a polyacrylamide-based electron-conducting redox hydrogel formed onto a vitreous carbon electrode (de Lumley-Woodyear et al., 1996). The DNA was covalently bound by carbodiimide coupling to the hydrazine functionality of the hydrogel surface. Improved results were obtained by using a 7 μm-diameter carbon fibre electrode (Caruana and Heller, 1999). In this case, the redox polymer was first electrophoretically deposited onto the microelectrode and then a carbodiimide-activated single-stranded probe was electrophoretically deposited and covalently attached to the redox-polymer film. The immobilisation process was monitored via the increased separation of ferrocenemethanol peak potentials.

The same group described the modification of carbon electrodes with a film of coelectrodeposited avidin and redox polymer. Incorporation of avidin into the electron-conducting hydrogel provided a platform to which biotinylated oligonucleotides could be promptly and simply attached (Campbell et al., 2002). This immobilisation procedure resulted in an higher probe hybridisation efficiency,

in comparison with that of previously reported covalently bound probes.

Wang et al. (1999) described the doping of nucleic acid probes within polypyrrole (PPy) films electropolymerised (by cyclic voltammetry scans) onto glassy carbon electrodes. Anionic oligonucleotide probes served as the sole charge-compensating counter ion during the growth of PPy films, retaining their hybridisation activity upon the entrapment.

2.2. Hybridisation with the target sequence

At present, the sensitivity of electrochemical DNA biosensors does not generally allow the direct analysis of genomic DNA. Almost all papers oriented towards the development of DNA hybridisation sensors deal with model, relatively short, oligonucleotide sequences. Only a few authors have reported the coupling of biosensors with a DNA amplification method (e.g. PCR) to obtain reliable measurements of clinical interest (Marrazza et al., 2000; Azek et al., 2000; Authier et al., 2001; Meric et al., 2002; Lucarelli et al., 2002). Direct, enzyme-based, detection of DNA or RNA with no PCR pre-amplification has rarely been reported (Downs et al., 1988; Wojciechowski et al., 1999).

Once re-association of two complementary strands has commenced, fast zippering enables the formation of successive base-pairs. The kinetics of the hybridisation reaction at solid surfaces have been reported to be slower than hybridisation in solution (Bunemann, 1982), due to the lower accessibility of the immobilised DNA probes.

Several variables affect the hybridisation event at the transducer-solution interface. These include salt concentration, temperature, the presence of accelerating agents, contact time and length of probe sequence.

The rate of hybridisation reaction is strongly dependent on the ionic strength: the kinetics dramatically increase upon raising the salt concentration to 3.2 M (Hames and Higgins, 1985). Wang et al. (1996a, 1997a,b) demonstrated that, by adding NaCl to the hybridisation solution, the analytical signal was greatly enhanced. It has to be pointed out that, in an indicator-free assay, a dramatic increase of the non-specific adsorption of the target oligonucleotide was observed at the electrode surface as a result of the increased ionic-strength (Lucarelli et al., 2002). Moreover, high salt concentrations stabilise mismatched duplexes. Thus such hybridisation conditions can not be applied when the detection of single-base mutations is required.

Temperature also strongly affects the hybridisation rate. Maximum kinetics are observed at 20–25 °C below the DNA–DNA duplex melting temperature (Hames and Higgins, 1985). From a practical point of view, stringent control of temperature and ionic strength (Millan et al., 1994; Caruana and Heller, 1999) during the assay facilitates discrimination between full matching and mismatching hybrids.

Wang et al. reported that the presence of the guanidine-HCl accelerator greatly facilitated the hybridisation reaction at solid surfaces (Wang et al., 1997a,b), with the hybridisation response rapidly increasing upon raising the accelerator concentration. Nevertheless, Lucarelli et al. observed a dramatic increase of the non-specific adsorption of the target oligonucleotide as a result of the addition of this compound to the hybridisation buffer.

A sequence length related effect on hybridisation kinetics was reported by Wang et al. (1996a). The reduced assay sensitivity associated with the use of a longer probe and target was attributed to steric hindrance influencing the hybridisation efficiency as well as to the slower target mass transport rate toward the probe-coated surface.

The rate of hybridisation is essentially independent of pH (Hames and Higgins, 1985). However, hybridisation experiments are, usually, carried out in phosphate (pH 7.0) or citrate (2 × SSC, pH 7.4) buffers. The interaction between the target sequence and the immobilised probe has been performed both by applying a potential (Wang et al., 1996a, 1997a,b) and under open circuit conditions (Marrazza et al., 2000); the incubation time ranged from 2 min to 1 h depending on the assay format.

In order to prevent the non-specific adsorption of unwanted solution constituents onto transducer surfaces, the use of blocking agents has been also proposed (de Lumley-Woodyear et al., 1996; Authier et al., 2001).

Biosensor selectivity has been assessed by monitoring their response in the presence of non-complementary sequences as well as mixtures containing the target sequence and various non-complementary strands or chromosomal DNAs (Wang et al., 1996a).

2.2.1. PCR samples

Due to the complexity of the matrix, PCR samples have to be diluted or purified before analysis. Double-stranded PCR products also have to be thermally denatured in order to obtain two single-stranded fragments, one of which is able to hybridise with the immobilised probe. The higher steric hindrance of the amplified target sequence reduced the hybridisation kinetic (Marrazza et al., 2000). Amplicon re-annealing also decreased the availability of the amplified target sequence and it was thus responsible of the lower hybridisation efficiency. In order to avoid amplicon re-annealing interference, Wojciechowski and co-workers proposed the conversion of double-stranded PCR products into single-stranded fragments by enzymatic digestion of interfering strand (Wojciechowski et al., 1999).

In the case of real samples analysis, biosensor selectivity has been assessed by monitoring their response in the presence of PCR blank or amplicons unrelated to the immobilised probe.

2.3. Labelling and electrochemical investigation of the surface

The hybridisation event is commonly detected via an increase in the current signal of an electroactive indica-

tor (that preferentially binds the double-stranded DNA), in conjunction with the use of enzyme or redox labels, or from other hybridisation-induced changes in electrochemical parameters. Several electroanalytical methods have been investigated for transducing the hybridisation event at carbon surfaces. Recent applications rely on the use of the most sophisticated, sensitive and fast techniques, including differential pulse voltammetry (DPV), square wave voltammetry (SWV) and potentiometric stripping analysis (PSA).

2.3.1. Intercalative compounds/groove binders

Early devices were based on the use of electroactive hybridisation indicators (Mikkelsen, 1996). Such indicators are small electroactive DNA-intercalating or groove-binding compounds, that possess much higher affinity for the surface-formed hybrid compared to the single-stranded DNA probe. Accordingly, the concentration of the indicator at the electrode surface increases when the hybridisation occurs, resulting in increased electrochemical response. In most cases however, the indicator association time has to be judiciously chosen. As the binding period increases, the hybridisation signal rises rapidly at first and then decreases. Such a decrease in the hybridisation response was attributed to the increased background peak signal and not to an actual decline of the absolute response (Wang et al., 1996a).

The minor groove binder $Co(bpy)_3^{3+}$ has been used by Mikkelsen's group for detecting the cystic fibrosis ΔF508 deletion sequence (Millan et al., 1994). Surface hybridisation, performed at 42 °C, allowed discrimination between the full complementary target sequence and the disease sequence (with three-base deletion). $Co(bpy)_3^{3+}$ has been also used by Wang et al. (1997a) to detect sequences related to a bacterial pathogen.

$Co(phe)_3^{3+}$ is another attractive indicator, due to its low peak potential and its greatly enhanced peak following the hybridisation (Wang et al., 1997c). This compound has been widely used to detect sequences related to bacterial (Wang et al., 1997b, 1998b) and viral pathogens (Erdem et al., 1999; Wang et al., 1996a). Chronopotentiometric detection of the $Co(phe)_3^{3+}$ or $Co(bpy)_3^{3+}$ markers resulted in detection limits of 0.05 μg/ml of oligonucleotide target sequence (Wang et al., 1997a).

Another useful redox-active indicator is the intercalating anthracycline antibiotic daunomycin. A detection limit of 0.01 μg/ml of target sequence was reported for the linear sweep voltammetric measurement of this label intercalated between the base pairs of a surface-formed hybrid (Hashimoto et al., 1994a). The differential pulse voltammetric measurement of the daunomycin peak potential shift (hybrid versus probe) was used by Mascini's group to detect the presence and the amount of complementary sequence (Marrazza et al., 1999). Nevertheless the shift was not reproducible and more than 4 μg/ml of complementary sequence were necessary to confirm the hybridisation. In contrast, the daunomycin-based chronopotentiometric DNA biosensor described by Marrazza et al. (2000) successfully detected apolipoprotein E genotypes in DNA samples extracted from whole human blood and amplified by PCR.

Ozsoz's group reported the use of methylene blue (MB) as hybridisation indicator. MB strongly associated with the free guanine bases of single-stranded DNA: the indicator peak decrease, following hybridisation, thus reflected the extent of duplex formation. The capability of MB to detect single-base mutations in short synthetic oligonucleotides (Erdem et al., 2000) and sequences related to TT and Hepatitis B virus (HBV) in amplified PCR samples (Meric et al., 2002) was also demonstrated with the use of DPV and SWV, respectively.

2.3.2. Enzyme labels

Enzyme labels have been widely used in affinity biosensors, particularly in immunosensors. The use of such a labels greatly amplify the hybridisation signals, offering considerable promise for ultrasensitive electrochemical detection of DNA hybridisation. A HRP-labelled DNA probe was used by Heller's group in combination with the covalent immobilisation of the target sequence (de Lumley-Woodyear et al., 1996). Upon hybridisation, the enzyme was electrically "wired" to the electrode surface and H_2O_2 was detected by amperometry. The themostable soybean peroxidase (SBP), covalently bound to target oligonucleotides, was also used by Heller's group for the enzyme-amplified amperometric detection of DNA hybridisation (Caruana and Heller, 1999). The accurate control of assay temperature allowed an easy discrimination between full-matching, single-base and four-base mismatching sequences. The hybridisation reaction was monitored in real time. About 34 000 copies of SBP-labelled hybrid could be detected in a 10 min assay. Heller's group also reported an enzyme-amplified amperometric sandwich test for RNA and DNA (Campbell et al., 2002). DNA or RNA sequences were simultaneously allowed to hybridise with a HRP-labelled detection sequence and the surface-confined probe. A decrease in the H_2O_2 electroreduction current indicated the presence of the analyte DNA or RNA in solution.

An electrochemical enzyme-amplified hybridisation assay for the detection of human cytomegalovirus DNA in PCR samples was described by Brossier's group (Azek et al., 2000). The biosensor format involved: (a) adsorption of denatured PCR products (target) onto the sensing area of a screen-printed carbon electrode; (b) hybridisation with a short, biotinylated, DNA probe; (c) hybrid labelling with a streptavidin-conjugated HRP; (d) differential pulse voltammetric detection of the enzyme-generated product. The electrochemical method showed a higher sensitivity compared with the agarose gel electrophoresis quantification and a microtiter plate-based spectrophotometric hybridisation assay; 3.6×10^5 copies/ml of amplified human cytomegalovirus DNA (406 bp) could be detected.

Alegret's group designed an enzyme-labelled amperometric genosensor based on a concept adapted from

classical dot-blot DNA analysis (Pividori et al., 2001). The analytical procedure consisted of five steps: (a) DNA target immobilisation by adsorption onto a nylon membrane; (b) hybridisation between the target DNA and the biotinylated probe; (c) hybrid labelling with a streptavidin-conjugated HRP; (d) integration of the modified membrane onto a graphite-polymer composite electrode transducer; (e) amperometric detection of H_2O_2. Sensor selectivity against a three-base mismatched oligonucleotide probe was demonstrated. The ssDNA-modified membrane could be conserved for at least 3 months (at 4 °C) without a significant decrease of performances.

Two different enzymatic approaches, for the detection and quantification of nucleic acids, have been reported by Wojciechowski et al. (1999). The first one allowed the detection of DNA or RNA with no PCR pre-amplification. It required two probes, specific for the target sequence, namely a biotinylated capture probe and a fluorescein-labelled detector probe. The target was first hybridised with both probes and captured on a neutravidin-modified sensor surface. The ternary complex was then incubated with an anti-fluorescein HRP conjugate and detected by measuring the product of the enzymatic reaction. The second approach could be used to detect and quantify double-stranded PCR products without a hybridisation step. This method relied on the specificity of a PCR amplification procedure. One PCR primer must be modified at the 5′-end with fluorescein and the second primer must be labelled at the 5′-end with biotin. Thus, the resulting PCR product could be directly captured on the sensor, reacted with an anti-fluorescein HRP conjugate and detected. Intermitted pulse amperometry was the novel electroanalytical technique developed for multichannel measurements involving a multiarray sensing platform. The assay selectivity was assessed by single-point mutation detection in the human factor V gene. Attomoles sensitivity (without PCR pre-amplification) or the detection of 50 target molecules (when PCR amplification was used) have been reported with a 15–30 min analysis time.

2.3.3. Direct covalent labelling of DNA target sequence

2.3.3.1. Gold nanoparticles. Brossier's group reported the use of thiol-modified DNA probes anchored (by chemisorption) to gold nanoparticles (Authier et al., 2001). The method was applied for the sensitive quantification of an amplified 406 bp DNA sequence related to human cytomegalovirus. The assay consisted of four steps: (a) passive adsorption of the denatured amplified target on the walls of a polystyrene microwell or the sensing area of a screen-printed electrode; (b) hybridisation with the oligonucleotide probe conjugated to a colloidal gold nanoparticle; (c) chemical or electrochemical oxidation of the gold; (d) anodic stripping voltammetric detection of the released Au^{III} ions at sandwich-type screen-printed microband electrodes. A detection limit of 6×10^6 copies of 406 bp amplified sequence was achieved with 30 min hybridisation time.

2.3.3.2. Osmium complex. Direct covalent labelling of DNA target sequence with osmium tetroxide, 2,2′-bipyridine has been proposed by Palecek's group (see Section 2.5).

2.4. Indicator-free approach

Increased attention has been given recently to direct, label-free electrochemical detection schemes. Such direct detection can be accomplished by monitoring the changes in some electrical parameter accrued from the hybridisation event. Such schemes greatly simplify the sensing protocol, as they eliminate the use of indicators. Moreover, the assay safety is improved, since the indicators are usually toxic or carcinogenic compounds. The first indicator-free scheme was introduced by Wang et al. (1996c). The hybridisation was detected by monitoring the decrease of the guanine peak of the immobilised probe (e.g. oligo $d(G)_{20}$), following the addition of the complementary oligo $d(C)_{20}$ target. However, this procedure was not applicable in most cases (e.g. for guanine containing targets). Such a limitation has been overcome by developing a new approach based on the use of inosine-modified (guanine-free) probes (Wang et al. 1998a, 2001a; Wang and Kawde, 2001; Lucarelli et al., 2002). The inosine moiety still forms a specific base-pair with the cytosine residue (Casegreen and Southern, 1994), but its electroactivity is about three orders of magnitude lower then that of guanine (Thorp, 1998). This results in a flat baseline (around +1.0 V) for the probe-modified electrode. The duplex formation was thus detected through the appearance of the guanine oxidation peak of the target sequence, following hybridisation. Detection limits of 0.12 μg/ml of oligonucleotide target sequence were reported for an indicator-free hybridisation biosensor based on the chronopotentiometric measurement of the target guanine signal (Wang et al., 1998a). The utility of such a label-free biosensor for the detection of single-base mutated oligonucleotide has been also demonstrated (Wang et al., 2001a; Wang and Kawde, 2001; Lucarelli et al., 2002). A similar biosensor was used by Lucarelli et al. to detect the sequences encoding for apolipoprotein E in DNA samples extracted from whole human blood and amplified by PCR.

A different label-free approach, based on doping nucleic-acid probes within conducting polymer films, was also proposed by Wang et al. (1999). Distinct transient hybridisation current peaks, with opposite directions in the presence of complementary and non-complementary DNA sequences, were obtained as a result of the changed conductivity of the host PPy network. Short hybridisation periods (a few seconds) allowed the detection of (μg/ml) target sequence.

2.5. Biomagnetic assays

The concept of the biomagnetic assay has been coupled, for instance, with the label-free detection approach (Wang et al., 2001a). The biosensor format involved: (a) capture

of the biotinylated, inosine-substituted DNA probe onto streptavidin-coated magnetic spheres; (b) hybridisation with a guanine-containing target; (c) magnetic separation of the surface-confined hybrid from the solution containing non-hybridised oligonucleotides; (d) alkaline denaturation of the hybrid (with the release of the target); (e) subsequent adsorptive stripping detection of the target guanine oxidation peak onto bare carbon electrodes. Detection limits of 0.10 μg/ml were reported for the label-free genomagnetic assay.

An enzyme-linked genomagnetic hybridisation assay was later proposed by Wang et al. (2002). The new protocol employed probe-modified magnetic beads able to hybridise (in solution) with a biotinylated DNA target. After hybrid labelling with streptavidin-conjugated alkaline phosphatase, the enzyme-generated α-naphtol was detected at bare screen-printed electrodes by using DPV. The bioanalytical assay was applied for the detection of short sequences related to breast-cancer BRCA1; a detection limit of 10 μg/l was achieved. Analogous probe-modified beads have been used to detect DNA hybridisation in connection with the precipitation of silver onto gold nanoparticle tags and subsequent electrochemical stripping detection of the dissolved silver onto thick-film carbon electrodes (Wang et al., 2001b). The procedure consisted of hybridisation of probe-modified beads with biotinylated target, which were subsequently coupled with streptavidin-coated 20 nm colloidal gold. Catalytic precipitation of silver onto gold nanoparticles greatly amplified the PSA signal; a detection limit of 0.2 ng/ml of BRCA1 breast cancer gene related sequences was reported.

Palecek et al. (2002a,b) described a magnetic beads-based, enzyme-linked immunoassay for the detection of the DNA hybridisation event. Paramagnetic beads, with covalently bound a $(dT)_{25}$ probe, were used for the hybridisation with target DNAs (synthetic 67-, 97-mer and PCR products) containing adenine stretches. Target DNAs were previously modified with osmium tetroxide, 2,2′-bipyridine and the immunogenic Os(bipy)-DNA adducts were determined by enzyme-linked immunoassay. The enzyme-generated α-naphtol was detected at bare carbon electrodes by using linear sweep voltammetry. Alternatively, Os(bipy)-modified targets were directly detected by measuring the osmium square wave voltammetric signal at pyrolytic graphite electrodes. A comparison between determination of the 67-mer target at carbon electrodes using: (a) the guanine oxidation signal (label-free detection of unmodified target); (b) direct determination of the Os(bipy)-DNA adduct and (c) its electrochemical immunoassay, showed immunoassay to be the most sensitive method (detection limit: 5 ng/ml). Amplicons of 226 bp were also successfully detected by immunoassay with high sensitivity and specificity.

In most cases the electrochemical DNA biosensors described are not reusable. Thermal or chemical regeneration of the surface-bound DNA probe often results in the desorption of the probe itself or in a severe decrease in the probe hybridisation efficiency (Wang et al., 1998b). Sometimes, the electrochemical measurement damages the DNA probe; moreover, electrode surface fouling due to the use of electroactive indicators is often irreversible (Wang et al., 1996a). To overcome the problems associated with this ineffective and time-consuming procedure, many groups have focused on the development of biosensors based on renewable pencil lead electrodes (Wang and Kawde, 2001) or “one-shot” disposable strips (Marrazza et al., 2000).

3. Gold electrodes

Almost all papers describing electrochemical genosensors are based on the use of commercially available electrodes obtained by sealing gold wires into plastic supports. Nevertheless, mechanical and chemical procedures to regenerate a new, bare, electrode surface after some measurement cycles are tedious and time consuming. A large number of uniform gold electrodes is required for the practical evaluation of real samples. The availability of disposable printed (Umek et al., 2001) and photolithographed (Hashimoto et al., 1998; Xu et al., 2001) gold electrodes greatly facilitates biosensing protocols. As in the case of disposable carbon strips, disposable gold electrodes also obviate the need for a regeneration step so that their future use for decentralised genetic testing can be easily envisioned.

3.1. Immobilisation of the DNA probe

The kinetics of nucleic acid hybridisation at solid surfaces are directly linked to the accessibility of the DNA probe. For this reason is desirable to attach nucleic acids covalently to the electrode surface through one of the ends of the DNA chain. Use of this approach can lead to probe structure flexibility with respect to change in its conformation when hybridisation take place (Yang et al., 1997). Random covalent binding of DNA to electrode surfaces, involving chemical modification of the bases, decreases the specificity of the recognition layer and therefore is not recommended (Palecek and Fojta, 2001). Self-assembly provide one of the most elegant approaches to obtain well defined and organised surfaces that can be an excellent platform for biosensor applications. General features of self-assembled monolayers (SAMs) onto gold surfaces have been summarised in several papers and reviews (Chidsey and Loiacono, 1990; Wink et al., 1997; Chaki and Vijayamohanan, 2002) and have been briefly covered in the following section. Even more elegant approaches involve the direct chemisorption of thiol-modified DNA probes onto gold surfaces.

3.1.1. Self-assembled monolayers onto gold surfaces: general features

The self-assembly of sulfur-containing molecules was characterised by Nuzzo and Allara almost 20 years ago (Nuzzo and Allara, 1983). Disulfides, sulfides and thiols coordinate very strongly onto a variety of metals, e.g. gold,

silver, platinum or copper. Nevertheless, gold is the most favoured because it is reasonably inert. The assumed reaction between a thiolate compound and a gold substrate is

$$RSH + Au \leftrightarrow RS\text{-}Au + e^- + H^+$$

Although dense monolayers assemble in less then 1 h, well ordered monolayers can take days to form (Bain et al., 1989).

An uncontaminated gold surface is important but not essential for the chemisorption; the high affinity of the thiol moiety for gold even displaces contaminants (Wink et al., 1997). Nevertheless, as a precaution, gold disk electrodes are, usually, mechanically polished with alumina, sonicated and etched in acidic or alkaline solutions to remove possible contaminants. This treatment is also important to produce a hydrophilic gold surface. Residual contaminants on bare gold can be removed by repeated cycling in diluted H_2SO_4 (Hashimoto et al., 1994b) or diluted KOH (Aoki et al., 2000) until stable voltammograms are obtained. Clean gold shows a characteristic anodic peak current near +1.1 V (versus SCE) and a single cathodic peak near +0.9 V in dilute sulfuric acid solutions (Finklea et al., 1987).

The mole ratio of a mixture of thiols in solution results in the same ratio at the self-assembled surface, thus indicating that the components do not phase segregate into islands (Bain and Whitesides, 1989). This interesting feature can be exploited to immobilise biomolecules in such a manner that steric hindrance between these molecules and their binding partners is avoided.

Monolayers are stable in the potential range from −400 to +1400 mV (vs SCE) in dilute sulfuric acid solutions (Finklea et al., 1987), thus allowing electrochemical applications. The careful selection of the terminal functionalities of the monolayers and the proper surface chemistry allows a tremendous flexibility in biosensor design.

3.1.2. Carbodiimide covalent coupling to preformed self-assembled monolayers

Three different types of thiols (hydroxyl, amino and carboxyl-terminated) were explored by Zhao et al. (1999) for optimal covalent immobilisation of DNA. Hydroxyl and amino-terminated SAMs were coupled to 5′-phosphate-end of DNA, while carboxyl-terminated one to the 3′-hydroxy-end of the nucleic acid by a water soluble carbodiimide. The influence of different terminal groups of SAMs was investigated by X-ray photoelectron spectroscopy (XPS) and cyclic voltammetry (CV). Both XPS and CV data confirmed that the hydroxyl-terminated SAM was more suitable for covalent attachment of DNA.

The carbodiimide covalent coupling of a 8-mer oligonucleotide to a previously self-assembled monolayer of cysteamine was described by Johansson and co-workers (Berggren et al., 1999).

A synthesised 24-mer oligonucleotide was covalently immobilised onto a self-assembled aminoethanethiol monolayer modified gold electrode, using a water-soluble carbodiimide by Fang and co-workers (Sun et al., 1998). In the presence of water-soluble carbodiimide reagent, the 5′-terminal phosphate of ssDNA formed a phosphoramidate bond with primary amino group of aminoethanethiol monolayer. Probe surface density was regulated by producing a mixed self-assembled monolayer of aminoethanethiol and hexylmercaptan. Probe immobilisation was confirmed by CV of $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ redox couple (see next section) and IR spectroscopy.

Oligonucleotide probes were covalently attached onto a 3-mercaptopropionic acid (MPA) self-assembled monolayer using NHS and a EDC by Ozsoz and co-workers (Kerman et al., 2002). The activation of the MPA-modified electrodes left the SAM surface terminated with a succinimide ester, which was susceptible to nucleophilic attack from free amino groups of DNA bases to form a peptide bond. The differential pulse voltammetric signal of MB was used to investigate the surface coverage.

Carbodiimide covalent coupling of a carboxylic acid-tagged oligonucleotide to cysteamine monolayer-modified gold electrodes has been reported by Willner and co-workers (Bardea et al., 1999a).

3.1.3. Direct self-assembly of alkanethiol-functionalised probes

Synthetic oligonucleotides can be modified with a 5′-terminal hexanethiol group or, alternatively, with a 3′-propanethiol group. The sulfur containing oligonucleotides are generally shipped as disulfides [DNA–$(CH_2)_6$–S–S–DMT or DNA–$(CH_2)_3$–S–S–$(CH_2)_3$–OH] to minimise the potential for oxidation resulting in oligo dimer formation. Prior to use, the disulfide linkage must be cleaved by using dithiothreitol (DTT); the excess of DTT and the protecting groups (dimethoxytrityl (DMT) and propanol) can be removed by size exclusion separation (Patolsky et al., 2001) or extraction with ethyl acetate (Xu et al., 1999). The use of non-deprotected, hexanol disulfides, 20-mer DNA probes has been, however, reported (Kertesz et al., 2000).

Chemisorption of thiol-derivatised, single-stranded DNA probes onto gold substrates has been extensively characterised by Herne and co-workers, by using XPS, ellipsometry, ^{32}P-radiolabeling, neutron reflectivity and electrochemical methods (Herne and Tarlov, 1997; Levicky et al., 1998; Steel et al., 1998). Similarly to long-chain alkanethiols, the full surface coverage by a monolayer of thiol-derivatised DNA (HS-ssDNA) can be attained in about 2 h. The ionic strength of HS-ssDNA solutions was found to have a profound effect on surface coverage, with chemisorption greatly enhanced at high salt concentrations. Authors postulated that intermolecular electrostatic repulsion between neighbouring DNA strands was minimised under the high ionic strength conditions, as the charged strands were better electrostatically shielded. More precise control over surface coverage and probe availability was achieved by creating mixed monolayers of the thiol-modified DNA probe and a spacer thiol, mercaptohexanol (MCH), by a two-step method, where first the gold substrate was exposed to a

micromolar solution of HS-ssDNA, followed by exposure to a millimolar solution of MCH. The post-treatment with MCH displaced non-specifically adsorbed HS-ssDNA molecules, e.g. those molecules that interacted with the surface also and only through nitrogen-containing bases. Neutron reflectivity measurements demonstrated that after post-treatment with MCH, DNA molecules "stand up" consistently with the primary attachment through the thiol-end group becoming accessible for specific hybridisation. Authors observed also that hybridisation of surface-bound HS-ssDNA was dependent on surface coverage; the complementary sequences could not access to surface bound probe when steric and electrostatic hindrance, arising from too tightly packed HS-ssDNA molecules, was too high. A hybridisation efficiency of about 100% was estimated for the optimised HS-ssDNA/MCH mixed monolayer.

Monolayers of thiol-modified DNA were reported to be electrochemically stable in a different potential window (−0.7 to +0.7 V versus SCE) compared to those of long-chain alkanethiols (Kelley and Barton, 1997). Hashimoto and co-workers pioneered the use of thiol-modified DNA probes for electrochemical sequence-specific gene detection almost 10 years ago (Hashimoto et al., 1994b). Nevertheless, most of recent publications follow Herne and Tarlov "guidelines". Self-assembly of thiol-modified probes (1–200 μM solutions) onto gold transducers was allowed to proceed for times ranging between 2 and 24 h. $MgCl_2$ containing DNA probe solutions were also used to achieve tight packing of the probe onto the electrode surface (Aoki et al., 2000; Boon et al., 2002). Improved sensitivity has been achieved, however, by producing low density probe-modified surfaces. Hashimoto et al., proposed the immobilisation of a synthetic 20-mer dsDNA and the subsequent regeneration of a free DNA probe by duplex alkaline denaturation (Hashimoto et al., 1998). The immobilisation of a 20-mer probe, with the subsequent masking of the unmodified gold regions with mercaptoethanol spacers, has been reported (Takenaka et al., 2000; Kertesz et al., 2000). A mixed monolayer of 10-mer cysteine-conjugated PNA probe and 6-mercapto-1-hexanol was used by Umezawa and co-workers to modified gold disk electrodes (Aoki et al., 2000).

Short thiols produce defective monolayers, which allow electrochemistry to occur at the underlying gold electrode. On the contrary, the detection scheme proposed by Barton and co-workers required the immobilisation of duplex monolayers so densely-packed that $[Fe(CN)_6]^{4-}$ was electrochemically silent (Kelley et al., 1999a).

A mixed self-assembled monolayer of alkanethiol-modified DNA capture probes, alkanethiol-terminated polyethylene glycols (that served as insulators for the surface) and highly conjugated, alkanethiol-terminated phenylethynyl molecular wires (that provided a pathway for electron transfer between labelled reporter probes and gold electrode surface), was described by Umek et al. (2001). An array of 14 gold electrodes (each of 250 or 500 μm diameter) in a printed circuit board, along with reference and auxiliary electrodes (to complete the electrochemical cell) was used to assemble such a monolayer.

The extent of surface coverage by the DNA probe can be conveniently determined by electrochemical methods. The most widely used is a method based on the cyclic voltammetric measurement of the $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ faradic response (Hashimoto et al., 1994b; Steel et al., 1998; Kelley and Barton, 1997; Xu et al., 2001; Nakayama et al., 2002). The peak currents of the redox couple decreased and the peak-to-peak separation (ΔE_p) increased when probe-modified electrodes were used and compared with bare electrodes. The shielding of the ferro–ferri-cyanide ions from the DNA-modified surface was attributed to a combination of physical coverage by the ssDNA and the electrostatic repulsion between the negatively charged redox couple ions and the DNA phosphate backbone (Yang et al., 1998).

Takenaka et al. (2000) reported an indirect quantification method based on the HPLC analysis of the excess of DNA remained unadsorbed.

3.1.4. Direct self-assembly of phosphorothioester-modified probes

A 16-mer probe, modified with five successive phosphothioate bonds from its 5′-end, was used to prepare the sensing interface by Ihara and co-workers (Nakayama et al., 2002). Sulphating was accomplished on the oxidation step in usual nucleotide synthesis. Five phosphothioates were introduced because the affinity of this group to gold was weaker than that of a thiol. The newly developed immobilisation chemistry was characterised by CV of ferro–ferri-cyanide redox couple, IR spectroscopy and quartz crystal microbalance (QCM). A hybridisation efficiency lower than 10% was observed. The 18-mer probes, containing five thiophosphate thymines, were also used by Willner and co-workers (Patolsky et al., 1999; Bardea et al., 1999b; Alfonta et al., 2001). The 18-mer oligonucleotides, modified by a phosphorothioate group either at its 3′ or both 3′ and 5′ terminals, were used by Hianik and co-workers to modify gold electrodes (Hianik et al., 2001). The sensors were subsequently exposed to 11-mercaptoundecanoic acid solution to mask unmodified gold regions.

3.2. Hybridisation with the target sequence

Variables affecting the hybridisation event at the transducer-solution interface have already been described with respect to graphite-based DNA hybridisation sensors. However, the use of different hybridisation accelerators, covalently-modified oligonucleotides or new strategies to detect single-point mutations are highlighted below.

Extreme hybridisation conditions were described by Xu et al. (2001). Besides high ionic strength hybridisation buffer (e.g. 5 × SSC (1 × SSC: 150 mmol/l NaCl; 15 mmol/l sodium citrate)), the use of 45% (v/v) of formamide and 5% (w/w) sodium dextran sulfate were reported. Formamide is known

to decrease the melting temperature of nucleic acid duplexes, thus allowing efficient hybridisation at lower temperatures (Hames and Higgins, 1985). On the other hand, dextran sulfate is an inert polymer that, by excluding the DNA from its own volume occupied in solution, effectively increase the concentration of DNA (Hames and Higgins, 1985).

Covalently-modified signaling probes have been used in different sandwich-type hybridisation assays (Umek et al., 2001; Yu et al., 2001; Nakayama et al., 2002). The remarkable similarity of melting profiles of unmodified and ferrocene-labelled duplexes indicated that the metal complex does not alter the hybridisation properties of the corresponding oligonucleotide (Yu et al., 2001). Only slight differences in the duplex melting curve were observed, also for anthraquinone-labelled DNAs (Kertesz et al., 2000).

A slow thermal annealing through the melting temperature of the duplex has been reported to increase the efficiency of the hybridisation process by Kertesz et al. (2000). A preliminary thermal denaturation of target sequences can also be necessary when DNA secondary structures are highly probable.

Reliable detection of mismatched base-pair is the main challenge for DNA hybridisation sensors. Mismatch discrimination has usually been achieved by relying on the different thermal stability of full matching and mismatching duplexes. Such temperature-dependent mismatch discrimination has been reported for DNA–DNA duplexes by Takenaka and co-workers (Miyahara et al., 2002), for PNA–DNA duplexes by Umezawa and co-workers (Aoki et al., 2000) and even for human amplified DNA genotyping by Umek et al. (2001). On the contrary, Barton and co-workers' approach was completely different. Both native and mutant test strands were allowed to associate with the capture probe under strongly hybridising conditions. Mismatch discrimination was then accomplished not by relying on the different thermodynamic stability of the duplexes, but measuring a change in electronic coupling within the base-pair stack (Kelley et al., 1999a; Boon et al., 2000).

Several authors have reported the electrochemical analysis of PCR-amplified real samples (Hashimoto et al., 1998; Miyahara et al., 2002; Umek et al., 2001; Xu et al., 2001). Willner and co-workers even reported the direct detection of specific genomic sequences with no PCR pre-amplification (Patolsky et al., 2001). Genomic DNA was simply extracted from human blood, digested in the presence of DNase, purified and diluted in the hybridisation buffer. The resulting solutions were used to detect the sequences related to the Tay-Sachs genetic disorder.

As already stated, amplicon sister strand re-annealing in solution competes with hybridisation to the capture probe at the electrode surface, thus decreasing the availability of the amplified target sequence. Umek et al. (2001) circumvented the problem by performing asymmetric PCR (e.g. using the forward and reverse primers in the 5:1 ratio), thus producing an excess of single-stranded target.

3.3. Labelling and electrochemical investigation of the surface

Labelling methods and electrochemical approaches to detect the hybridisation event are similar to those described for use with graphite-based sensors. The use of faradic impedance spectroscopy for probe biorecognition, however, was a major innovation.

3.3.1. Intercalative compounds/groove binders

Hashimoto et al. (1994b) reported the detection of a 4.2 kbp linearised plasmid carrying the *Pst*I fragment of oncogene v-*myc*. The linear-sweep voltammetric measurement of the redox-active, minor groove binder, Hoechst 33258, allowed a detection limit of 10^{-13} g/ml of target sequence. The same group also reported the indicator-based (Hoechst 33258) detection of HBV genome DNA in clinical samples (Hashimoto et al., 1998). The sensitivity of the disposable DNA sensor was first estimated using a recombinant plasmid pYRB259 containing a *Bam*HI fragment of HBV DNA. A detection threshold of 10^4–10^5 copy/ml was reported. The DNA sensor was then applied for the detection of HBV DNA in samples extracted from patients's era. The concentrations determined by the electrochemical method were in good agreement with those obtained by a conventional nucleic acid quantification method (competitive polymerase chain reaction).

Umezawa and co-workers developed a new ion-channel protocol for indirect biosensing of DNA hybridisation (Aoki et al., 2000). The system relied on the electrostatic suppression of the cyclic voltammetric signal of the diffusing hexacyanoferrate II redox marker, due to the hybridisation between the neutral PNA probe and the negatively-charged target DNA. High specificity towards mismatch oligonucleotides was observed and the sensitivity (micromolar level) required some improvement.

A new strategy for the electrochemical detection of single-base mismatches, based on DNA-mediated electron transfer, was developed by Barton's group (Kelley et al., 1999a). The presence of mismatches within the immobilised duplexes, caused a striking decrease in the electrochemical response of different redox-active intercalators (daunomycin, MB and $[Ir(bpy)(phen)(phi)]^{3+}$), non-covalently bound to the top of densely-packed DNA-modified gold surfaces. Mismatch detection was accomplished irrespective of DNA sequence and mismatch identity. In order to increase the inherent sensitivity of the assay, the redox reaction of the intercalated MB was electrocatalytically enhanced by using potassium ferricyanide as the solution substrate. Such electrocatalytic approach both increased the sensitivity of mismatch detection and provided larger absolute signals. The sensitivity of this elegant assay depended on electronic coupling within the base-pair stack, rather than on the thermodynamics of base pairing. Thus, gold electrodes, modified with pre-assembled DNA duplexes, were used to detect the eight possible single-base mismatches (including thermodynami-

cally stable GT and GA mismatches) as well as "hot spots" mutations in the human *p53* tumour suppressor gene (Boon et al., 2000). Furthermore, many naturally occurring DNA lesions (8-oxo-adenine, 5,6-dihydro thymine and deoxyuracil) could be detected, despite their relatively high thermodynamic stability and minor modifications induced in DNA duplexes. The applicability of this technology in a chip-based format was also demonstrated. About 10^8 target molecules were detected on a 30 μm diameter gold electrode.

Osmium-5,6-dimethyl-1,10-phenanthroline, $[Os(5,6\text{-dmphen})_3]^{2+}$, has been proposed as the hybridisation indicator by Maruyama et al. (2001) in conjunction with the Osteryoung SWV pulse technique. The 20-mer target DNA was detected linearly in the range 6.9×10^{-10} to 6.9×10^{-5} g/ml. No detectable increase in the indicator response was measured when the mismatched sequence was incubated with the probe-modified surface.

Daunomycin, as the electroactive intercalator, was directly added in the target sequence solutions by Fang and co-workers (Sun et al., 1998). Thus, hybridisation and indicator binding occurred simultaneously. Target sequence detection was achieved by measuring the cathodic peak current of daunomycin by means of linear sweep voltammetry. The signal was linearly related to the concentration of target sequence between 0.1 and 0.1 ng/ml, with a detection limit of 30 pg/ml. The DNA sensor was also successfully used for the specific detection of a model target gene (400 bp yAL_3).

The decrease in the magnitude of the voltammetric reduction signal of MB was used by Ozsoz and co-workers (Kerman et al., 2002) to detect the hybridisation reaction. Peak currents were found to decrease in the order: probe-modified, one base mismatched hybrid-modified and hybrid-modified electrodes. The sensitivity of the method, in the ppm range, was, however, quite poor.

Beside "classical" indicators, new electroactive indicators have been developed for attaining higher sensitivity. Two classes of intercalators, bis- and threading intercalators, were particularly interesting as new redox indicators. The natural occurring bis-intercalator echinomycin, an antibiotic and antitumor agent, was reported to bind dsDNA more tightly than usual indicators (Palecek and Fojta, 2001).

Takenaka's group reported that ferrocenyl naphtalene diimide (FND) intercalates to DNA duplex with a threading-type mode, where its two substituents are projecting out in the major and minor groove simultaneously (Miyahara et al., 2002). The substituents serve as anchors to prevent the dissociation of the ligand from double-stranded DNA; this compound displays, on the other hand, a negligible affinity for the DNA probe, since the threading mode of interaction is not advantageous for single-stranded DNA. The differential pulse voltammetric detection of FND allowed a detection limit of 10 zmol of 20-mer target sequence and 1 fmol of a linearised plasmid DNA carrying a part of the yeast choline transport gene (Takenaka et al., 2000). The method was also applied for the detection of DNA extracted for the peripheral whole blood and amplified by PCR (Miyahara et al., 2002). The sensitivity of the method was limited by the high indicator background signal attributed to the pure diffusion of FND toward the electrode surface. Single nucleotide polymorphisms in DNA fragments (278 bp) of exon 4 of cancer repression gene *p53* were identified with only modest precision. The higher steric hindrance of the amplified target sequence, amplicon re-annealing and the inhomogeneity of commercial gold electrodes were considered responsible for such poor accuracy.

Chambers and co-workers proposed a different approach for the detection of DNA hybridisation based on the surface tritation of DNA-modified gold electrodes with a thiol-tethered anthraquinone (Kertesz et al., 2000). In this method, a thiol-modified 20-mer probe or the corresponding DNA duplex were immobilised onto bare gold electrodes, treated with the MCH spacer and, finally, with a thiol-tethered anthraquinone. Subsequent exposure of surface-confined nucleic acids to pure, warm buffer denatured the double-stranded DNA, thus resulting in an increased surface area for a further reaction with the thiol-tethered indicator. A marked increase in the anthraquinone signal, observed only in the case of duplex-modified electrodes, allowed easy differentiation between ss- and dsDNA.

3.3.2. Enzyme labels

A biosensing method, based on the enzyme-amplified detection of hybridisation reaction, has been reported by Xu et al. (2001). Probe-modified gold electrodes were first hybridised to a biotinylated complementary sequence. The alkaline-phosphatase enzyme, conjugated with avidin, was then coupled to the hybrid via the avidin–biotin complex. In the last step, the enzymatic cleavage of the electro-inactive substrate (naphthyl phosphate) produced α-napthol, which could be detected by DPV. Significant selectivity against mismatched oligonucleotide sequences was achieved. The practical usefulness of the genosensor was also demonstrated with PCR products (566 bp from the HBV DNA plasmid) analysis. A detection limit of 0.22 nmol/l was reported.

A novel method for the electronic transduction of DNA hybridisation, based on faradic impedance spectroscopy, was reported by Willner and co-workers (Bardea et al., 1999b). Impedance spectra were recorded in the presence of $[Fe(CN)_6]^{3-/4-}$ redox probe. As the negatively charged DNA probe and hybrid were immobilised and formed, respectively, at the electrode surface, the electrostatic repulsion between them and the negatively charged redox probe introduced an electron-transfer resistance that could be detected by faradic impedance spectroscopy. A further barrier for the interfacial electron transfer was introduced by conjugating avidin molecules to the biotin-labelled target, thus confirming and amplifying the hybridisation event. Applicability for the detection of mutation relevant to the Tay-Sachs genetic disorder was demonstrated.

A further method to amplify the electronic transduction of hybridisation event involved the use of cytochrome *c*, a versatile electron-transfer hemoprotein mediator for redox-active enzymes (Bardea et al., 1999a). Cytochrome *c* is a positively charged protein that is effectively attracted by negatively charged interfaces. The molecule itself lacked direct electrical communication with the electrode support and also the low interfacial potential at probe-modified surfaces had a limited effect on the electron transfer. On the contrary, the electrostatic attraction by the double-stranded DNA, aligned the heme center of cytochrome *c* in respect to the electrode surface, thus dramatically enhancing the electrical communication. Preliminary results showed the easy differentiation of normal and mutated genes carrying the Tay-Sachs genetic disorder.

A sandwich-type, enzyme-linked, hybridisation assay was also reported by Willner and co-workers. The surface-linked, thiol-modified, capture probe 1 was reacted with the complex: target DNA-biotinylated capture probe 2. Ternary complex formation introduced a barrier to interfacial electron-transfer of $[Fe(CN)_6]^{3-/4-}$ redox probe that could be readily detected by faradic impedance spectroscopy (Patolsky et al., 1999) and/or chronopotentiometry (Alfonta et al., 2001). Binding of an avidin-HRP conjugate to the double-stranded superstructure, further increased the electron-transfer resistance. A strong insulation of the electrode surface was achieved by the HRP-mediated oxidation of 4-chloro-1-naphtol by H_2O_2 that yielded an insoluble product. Thus, the precipitation of this product was used to confirm and amplify the sensing process of target DNA. Easy discrimination between normal and mutated gene characteristic of the Tay-Sachs genetic disorder was achieved with a detection limit of 2×10^{-2} μg/ml.

Willner and co-workers also reported the detection of single-base mutations in real DNA samples with no PCR pre-amplification (Patolsky et al., 2001). A thiolated probe was designed to be complementary to the target DNA as far as one base preceding the mutation site. After hybridising the target DNA, normal or mutant, with the sensing probe, the resulting assembly was reacted with a biotinylated nucleotide, complementary to the mutation site, in the presence of DNA polymerase. The labelled nucleotide was, thus, coupled only to the double-stranded assembly that included the mutant site. Subsequent binding of avidin-conjugated alkaline phosphatase to the biotinylated duplex, catalysed the oxidative hydrolysis of 5-bromo-4-chloro-3-indolyl phosphate to an insoluble derivative, thus providing a means to confirm and amplify the detection of mutant. Faradic impedance spectroscopy was employed as the electroanalytical method, with a detection limit of 1×10^{-14} mol/ml of target oligonucleotide. The sensitivity of the method enabled even the quantitative analysis of polymorphic DNA samples (related to the Tay-Sachs genetic disorder) with no PCR pre-amplification. Target mutants were detected within the enormous mixture of genomic DNA fragments, revealing impressive selectivity of the sensing interface.

3.3.3. Direct covalent labelling of DNA sequence

3.3.3.1. Daunomycin. Barton's group demonstrated that redox-active intercalators, bound at discrete sites within the helix of a 15-mer duplex immobilised onto a gold electrode, exhibited remarkably fast electron transfer over long distances (Kelley et al., 1999b). Efficient reduction of daunomycin (cross-linked to the two amino group of guanine) was observed regardless of its position along the 15 base-pair sequence. Charge migration through DNA helix was found to be completely suppressed by the presence of a single-base mismatch.

3.3.3.2. Ferrocene. The use of ferrocene-labelled oligonucleotides for sequence-specific detection, mismatch discrimination and gene expression monitoring, in sandwich-type assays has been reported by Umek et al. (2001). This technology is actually used in a new, commercially available, hand-held device, the CMS eSensor™ system of Motorola Inc. Unlabelled nucleic acid targets were immobilised onto the electrode surface through sequence-specific hybridisation with a thiol-modified capture probe. A signaling probe, containing adenine residues modified with a ferrocene group, was designed to hybridise in a region of the target adjoining the capture probe binding site. The self-assembled surface allowed electron-transfer only from ferrocene groups electronically coupled to the gold (e.g. those involved in the ternary complex), while insulating the electrode from unbound signaling probes. Thus, a one-step hybridisation/detection protocol was developed, eliminating the need to remove the excess of reagents before the interrogation of the electrode surface. AC voltammetry was used as the electroanalytical method. Insertion/deletion polymorphism in intron 16 of the angiotensin converting enzyme (*ACE*) gene was detected in 192 bp DNA fragments. Amplicons, obtained by asymmetric PCR (A-PCR) from human *Hfe* gene, were also readily genotyped by virtue of single-base mismatches lower thermal stability. The sequences of five apoptosis-regulated genes (*fas*, *p53*, *bax*, *p21* and *bcl-2*) obtained by reverse transcription from total white blood cell RNA, subsequently amplified by A-PCR, were finally detected on the electrode array. Capture and signaling probes were carefully designed, in order to minimise the chance of their direct interaction and cross-hybridisation between amplicons and unrelated probes. Different PCR mixtures could be pooled loosing sensitivity but not selectivity.

To increase the rapidity and the accuracy of single-base mismatch detection, two types of metal-containing signaling probes (one specific for the wild-type target, the other for the mutant), with two different redox potentials, were also developed (Yu et al., 2001).

A sandwich-type hybridisation assay, based on the use of a ferrocene-conjugated DNA probe, has been also described by Ihara and co-workers (Nakayama et al., 2002). By using this method and DPV as the electrochemical technique, the authors successfully detected single-base mutations in a

19-mer target sequence. Nevertheless, sensor development was still in the earlier stage. The poor reproducibility was attributed to some irregularities in the procedure for immobilising the probe and to the inhomogeneity of commercial gold electrodes.

3.3.3.3. Anthraquinone. The 20-mer oligonucleotides, modified with anthraquinone groups tethered to the 2′-O position of a uridine nucleotide have also been considered for electrochemical detection of DNA hybridisation (Kertesz et al., 2000).

3.3.4. Label-free approach

Johansson's group demonstrated that the decrease in capacitance of a probe-modified gold electrode, accrued from the hybridisation to the complementary strand and the corresponding displacement of solvent molecules from the surface, can be used for monitoring with high sensitivity and speed the hybridisation event (Berggren et al., 1999). In hybridisation studies performed in a flow-through cell, as few as 25 molecules of single-stranded, 179 bp cytomegalovirus target sequence could be detected. Negative controls, such as double-stranded cytomegalovirus target sequence, single-stranded 104 bp hepatitis B virus sequence and single-stranded 207 bp tyrosinase mRNA sequence, all gave lower responses. Despite the extremely low detection limits reported, the authors admitted the poor specificity and reproducibility for such capacitive genosensor.

Hianik et al. (2001) tested the possibility of label-free amperometric detection of DNA hybridisation. The influence of DNA probe orientation (one point attachment versus two points attachment) was also studied. Addition of complementary oligonucleotide resulted in an increase of conductivity for the sensor containing the single-stranded probe oriented perpendicular to the gold support (phosphorothioate group at 3′-end), while the signal decreased in the case of DNA probe oriented parallel (phosphorothioate groups at both 3′ and 5′ terminals). Increase in conductivity was attributed to the increased exposure of sensor surface to the electrolyte solution after the formation of a vertical and rigid duplex; the decrease, to the stepwise coverage of the sensor surface and the reduced defects in sensor insulation. Addition of non-complementary chain resulted in a slight decrease or no change of sensor conductivity.

4. Conclusions

Over the past decade, enormous progresses have been made towards the development of electrochemical DNA biosensors. Such devices are of considerable interest due to their promise for obtaining sequence-specific information in a faster, simpler and cheaper manner compared to traditional nucleic acid assays.

To date, the widespread use of graphite-based sensors has been justified by their low cost (due to the well developed mass production of screen-printing) and, from the electrochemical point of view, the wide useful potential window. Despite these favourable aspects of carbon surfaces, only complicated immobilisation chemistries can lead to highly reactive DNA probe layers. Therefore, effective immobilisation procedures such as those reported by Mikkelsen's and Heller's groups present practical disadvantages. In general, the immobilisation of DNA probe molecules onto the electrode surface with controlled quality, coverage and orientation is still the most critical aspect of genosensor design.

On the contrary, self-assembly of terminally thiol-labelled oligonucleotides offers a simple and elegant method to modify gold surfaces. This approach, extensively characterised by Herne, Tarlov and co-workers, provides a unique probe structure flexibility that allows nearly a 100% hybridisation efficiency. The relatively low cost of disposable printed and photolithographed gold electrodes is making these kind of sensors even more attractive.

The use of electroactive hybridisation indicators has shown to have severe limitations. Almost all the proposed indicators bound not only the double-stranded DNA but also the single-stranded form, generally via electrostatic interaction with the phosphate groups of the DNA backbone. Hashimoto and co-workers underlined this problem. The length of the DNA target influenced the indicator response since it was bound not only to the double-stranded part of the hybrid DNA (20 bp), but also to the single-stranded target. Moreover, the sensitivity of these methods was usually reduced by the high indicators background signal attributed to their pure diffusion toward the electrode surface. Also the poor chemical stability and the (geno)-toxicity of most of the indicators can not be ignored.

Reliable detection of mismatched base-pair is the main challenge for DNA hybridisation sensors. Despite the limits stated above for indicator-based schemes, the approach proposed by Barton's group and based on electronic coupling within the base-pair stack, has to be mentioned because of its elegance.

To date, the use of enzyme labels to amplify the hybridisation signal seems to be the most promising approach for ultrasensitive detection of specific DNA sequences, since it enables even the quantitative analysis of polymorphic DNA samples with no PCR pre-amplification.

The use of magnetic beads has substantially solved the problem of the non-specific adsorption affecting several biosensing protocols. Separation of the biorecognition event from the transduction step (at beads and electrode surfaces, respectively) is, however, in contrast with the general concept of biosensor.

It is generally appreciated that the development of electrochemical genosensors is still at an early stage. Particular attention has still be given to the major challenges of mismatch discrimination and signal amplification. Integration of multiple sensors on a single miniaturised platform

(DNA microarray) should lead to significant advantages in terms of reliability, cost, speed and simplicity of the detection of specific DNA sequences. Future research must be focused on the development of simple analytical procedures, envisioning the large-scale use of electrochemical genosensors in hospitals and in medical practice in general. Inexpensive and powerful electrochemical devices could play an important role in the future of molecular diagnostics.

References

Alfonta, L., Bardea, A., Khersonsky, O., Katz, E., Willner, I., 2001. Chronopotentiometry and faradic impedance spectroscopy as signal transduction methods for the biocatalytic precipitation of an insoluble product on electrode supports: routes for enzyme sensors, immunosensors and DNA sensors. Biosens. Bioelectr. 16, 675–687.

Aoki, H., Bühlmann, P., Umezawa, Y., 2000. Electrochemical detection of a one-base mismatch in an oligonucleotide using ion-channel sensors with self-assembled PNA monolayers. Electroanalysis 12 (16), 1272–1276.

Authier, L., Grossiord, C., Brossier, P., 2001. Gold nanoparticles-based quantitative electrochemical detection of amplified human cytomegalovirus DNA using disposable microband electrodes. Anal. Chem. 73, 4450–4456.

Azek, F., Grossiord, C., Joannes, M., Limoges, B., Brossier, P., 2000. Hybridisation assay at a disposable electrochemical biosensor for the attomole detection of amplified human cytomegalovirus DNA. Anal. Biochem. 284, 107–113.

Bain, C.D., Whitesides, G.M., 1989. Formation of monolayers by coadsorption of thiols on gold: variation in the length of the alkyl chain. J. Am. Chem. Soc. 111, 7164–7175.

Bain, C.D., Troughton, E.B., Tao, Y.-T., Evall, J., Whitesides, G.M., Nuzzo, R.G., 1989. Formation of monolayer films by the spontaneous assembly of organic thiols from solution onto gold. J. Am. Chem. Soc. 111, 321–335.

Bardea, A., Dagan, A., Willner, I., 1999a. Amplified electronic transduction of oligonucleotide interactions: novel routes for Tay-Sachs biosensors. Anal. Chim. Acta 385, 33–43.

Bardea, A., Patolsky, F., Dagan, A., Willner, I., 1999b. Sensing and amplification of oligonucleotide–DNA interactions by means of impedance spectroscopy: a route to a Tay-Sachs sensor. Chem. Comm., 21–22.

Berggren, C., Stalhandske, P., Brundell, J., Johansson, G., 1999. A feasibility study of a capacitive biosensor for direct detection of DNA hybridisation. Electroanalysis 11 (3), 156–160.

Boon, E.M., Ceres, D.M., Drummond, T.G., Hill, M.G., Barton, J.K., 2000. Mutation detection by electrocatalysis at DNA-modified electrodes. Nat. Biotechnol. 18, 1096–1100.

Boon, E.M., Salas, J.E., Barton, J.K., 2002. An electrical probe of protein–DNA interactions on DNA-modified surfaces. Nat. Biotechnol. 20, 282–286.

Bunemann, T., 1982. Immobilisation of denaturated DNA to macroporous supports: II. Steric and kinetic parameters of heterogeneous hybridisation reactions. Nucleic Acids Res. 10, 7181–7196.

Cai, X., Rivas, G., Farias, P.A.M., Shiraishi, H., Wang, J., Palecek, E., 1996. Evaluation of different carbon electrodes for adsorptive stripping analysis of nucleic acids. Electroanalysis 8 (8-9), 753–758.

Campbell, C.N., Gal, D., Cristler, N., Banditrat, C., Heller, A., 2002. Enzyme-amplified amperometric sandwich test for RNA and DNA. Anal. Chem. 74, 158–162.

Caruana, D.J., Heller, A., 1999. Enzyme-amplified amperometric detection of hybridisation and of a single-base pair mutation in an 18-base oligonucleotide on a 7-μM-diameter microelectrode. J. Am. Chem. Soc. 121, 769–774.

Casegreen, S., Southern, E., 1994. Studies on base pairing properties of deoxyinosine by solid phase hybridisation to oligonucleotides. Nucleic acids Res. 22 (2), 131–136.

Chaki, N.K., Vijayamohanan, K., 2002. Self-assembled monolayers as a tunable platform for biosensor applications. Biosens. Bioelectron. 17, 1–12.

Chidsey, C.E.D., Loiacono, D.N., 1990. Chemical functionality in self-assembled monolayers: structural and electrochemical properties. Langmuir 6, 682–691.

de Lumley-Woodyear, T., Campbell, C.N., Heller, A., 1996. Direct enzyme-amplified electrical recognition of a 30-base model oligonucleotide. J. Am. Chem. Soc. 118, 5504–5505.

Downs, M.E.A., Warner, P.J., Turner, A.P.F., 1988. Optical and electrochemical detection of DNA. Biomaterials 9, 66–70.

Erdem, A., Kerman, K., Meric, B., Akarca, U.S., Ozsoz, M., 1999. DNA electrochemical biosensor for the detection of short DNA sequences related to the hepatitis B virus. Electroanalysis 11 (8), 586–588.

Erdem, A., Kerman, K., Meric, B., Akarca, U.S., Ozsoz, M., 2000. Novel hybridisation indicator methylene blue for the electrochemical detection of short DNA sequences related to the hepatitis B virus. Anal. Chim. Acta 422, 139–149.

Finklea, H.O., Avery, S., Lynch, M., Furtsch, T., 1987. Blocking oriented monolayers of alkyl mercaptans on gold electrodes. Langmuir 3, 409–413.

Hames, B.D., Higgins, S.J., 1985. Nucleic Acid Hybridisation—A Practical Approach. Oxford University Press, Oxford, p. 78.

Hashimoto, K., Ito, K., Ishimori, Y., 1994a. Novel DNA sensor for electrochemical gene detection. Anal. Chim. Acta 286 (2), 219–224.

Hashimoto, K., Ito, K., Ishimori, Y., 1994b. Sequence-specific gene detection with a gold electrode modified with DNA probes and an electrochemically active dye. Anal. Chem. 66, 3830–3833.

Hashimoto, K., Ito, K., Ishimori, Y., 1998. Microfabricated disposable DNA sensor for detection of hepatitis B virus DNA. Sens. Actuators B 46, 220–225.

Herne, T.M., Tarlov, M.J., 1997. Characterisation of DNA probes immobilised on gold surfaces. J. Am. Chem. Soc. 119, 8916–8920.

Hianik, T., Gajdos, V., Krivanek, R., Oretskaya, T., Metelev, V., Volkov, E., Vadgama, P., 2001. Amperometric detection of DNA hybridisation on a gold surface depends on the orientation of oligonucleotide chains. Bioelectrochemistry 53, 199–204.

Kelley, S.O., Barton, J.K., 1997. Electrochemistry of methylene blue bound to a DNA-modified electrode. Bioconjug. Chem. 8, 31–37.

Kelley, S.O., Boon, E.M., Barton, J.K., Jakson, N.M., Hill, M.G., 1999a. Single-base mismatch detection based on charge transduction through DNA. Nucleic Acids Res. 27 (24), 4830–4837.

Kelley, S.O., Jakson, N.M., Hill, M.G., Barton, J.K., 1999b. Long-range electron transfer through DNA films. Angew. Chem. Int. Ed. 38 (7), 941–945.

Kerman, K., Ozkan, D., Kara, P., Meric, B., Gooding, J.J., Ozsoz, M., 2002. Voltammetric determination of DNA hybridisation using methylene blue and self-assembled alkanethiol monolayer on gold electrode. Anal. Chim. Acta 462, 39–47.

Kertesz, V., Whittemore, N.A., Chambers, J.Q., McKinney, M.S., Baker, D.C., 2000. Surface tritation of DNA-modified gold electrodes with a thiol-tethered anthraquinone. J. Electroanal. Chem. 493, 28–36.

Levicky, R., Herne, T.M., Tarlov, M.J., Satija, S.K., 1998. Using self-assembly to control the structure of DNA monolayers on gold: a neutron reflectivity study. J. Am. Chem. Soc. 120, 9787–9792.

Lucarelli, F., Marrazza, G., Palchetti, I., Cesaretti, S., Mascini, M., 2002. Coupling of an indicator-free electrochemical DNA biosensor with polymerase chain reaction for the detection of DNA sequences related to the apolipoprotein E. Anal. Chim. Acta 469, 93–99.

Marrazza, G., Chianella, I., Mascini, M., 1999. Disposable DNA electrochemical DNA sensor for hybridisation detection. Biosens. Bioelectron. 14, 43–51.

Marrazza, G., Chiti, G., Mascini, M., Anichini, M., 2000. Detection of human apolipoprotein E genotypes by DNA electrochemical biosensor coupled with PCR. Clin. Chem. 46 (1), 31–37.

Maruyama, K., Motonaka, J., Mishima, Y., Matsuzaki, Y., Nakabayashi, I., Nakabayashi, Y., 2001. Detection of target DNA by electrochemical method. Sens. Actuators B 76, 215–219.

Meric, B., Kerman, K., Ozkan, D., Kara, P., Erensoy, S., Akarca, U.S., Mascini, M., Ozsoz, M., 2002. Electrochemical DNA biosensor for the detection of TT and Hepatitis B virus from PCR amplified real samples by using methylene blue. Talanta 56, 837–846.

Mikkelsen, S.R., 1996. Electrochemical biosensors for DNA sequence detection. Electroanalysis 8 (1), 15–19.

Millan, K.M., Mikkelsen, S.R., 1993. Sequence-selective biosensor for DNA based on electroactive hybridisation indicators. Anal. Chem. 65, 2317–2323.

Millan, K.M., Spurmanis, A.J., Mikkelsen, S.R., 1992. Covalent immobilization of DNA onto glassy carbon electrodes. Electroanalysis 4, 929–932.

Millan, K.L., Saraullo, A., Mikkelsen, S.R., 1994. Voltammetric DNA biosensor for cystic fibrosis based on a modified carbon paste electrode. Anal. Chem. 66, 2943–2948.

Miyahara, H., Yamashita, K., Kanai, M., Uchida, K., Takagi, M., Kondo, H., Takenaka, S., 2002. Electrochemical analysis of single nucleotide polymorphisms of *p53* gene. Talanta 56, 829–835.

Nakayama, M., Ihara, T., Nakano, K., Maeda, M., 2002. DNA sensors using a ferrocene-oligonucleotide conjugate. Talanta 56, 857–866.

Nuzzo, R.G., Allara, D.L., 1983. Adsorption of bifunctional organic disulfides on gold surfaces. J. Am. Chem. Soc. 105, 4481–4483.

Palecek, E., 2002. Past, present and future of nucleic acids electrochemistry. Talanta 56, 809–819.

Palecek, E., Fojta, M., 2001. Detecting DNA hybridisation and damage. Anal. Chem. 73, 74A–83A.

Palecek, E., Kizek, R., Havran, L., Billova, S., Fojta, M., 2002a. Electrochemical enzyme-linked immunoassay in a DNA hybridisation sensor. Anal. Chim. Acta 469, 73–83.

Palecek, E., Fojta, M., Jelen, F., 2002b. New approaches in the development of DNA sensors: hybridisation and electrochemical detection of DNA and RNA at two different surfaces. Bioelectrochemistry 56, 85–90.

Patolsky, F., Katz, E., Bardea, A., Willner, I., 1999. Enzyme-linked amplified electrochemical sensing of oligonucleotide-DNA interactions by means of the precipitation of an insoluble product and using impedance spectroscopy. Langmuir 15, 3703–3706.

Patolsky, F., Lichtenstein, A., Willner, I., 2001. Detection of single-base DNA mutations by enzyme-amplified electronic transduction. Nature Biotechnol. 19, 253–257.

Pividori, M.I., Merkoci, A., Alegret, S., 2000. Electrochemical genosensor design: immobilisation of oligonucleotides onto transducer surfaces and detection methods. Biosens. Bioelectron. 15, 291–303.

Pividori, M.I., Merkoçi, A., Alegret, S., 2001. Classical dot-blot format implemented as an amperometric hybridisation genosensor. Biosens. Bioelectron. 16, 1133–1142.

Schülein, J., Graßl, B., Krause, J., Schulze, C., Kugler, C., Müller, P., Bertling, W.M., Hassmann, J., 2002. Solid composite electrodes for DNA enrichment and detection. Talanta 56, 875–885.

Steel, A.B., Herne, T.M., Tarlov, M.J., 1998. Electrochemical quantification of DNA immobilised on gold. Anal. Chem. 70, 4670–4677.

Sun, X., He, P., Liu, S., Ye, J., Fang, Y., 1998. Immobilisation of single-stranded deoxyribonucleic acid on gold electrode with self-assembled aminoethanethiol monolayer for DNA electrochemical sensor application. Talanta 47, 487–495.

Takenaka, S., Yamashita, K., Takagi, M., Uto, Y., Kondo, H., 2000. DNA sensing on a DNA probe-modified electrode using ferrocenylnaphthalene diimide as the electrochemical active ligand. Anal. Chem. 72, 1334–1341.

Thorp, H.H., 1998. Cutting out the middleman: DNA biosensors based on electrochemical oxidation. Tibtech 16, 117–121.

Umek, R.M., Lin, S.W., Vielmetter, J., Terbrueggen, R.H., Irvine, B., Yu, C.J., Kayyem, J.F., Yowanto, H., Blackburn, G.F., Farkas, D.H., Chen, Y.-P., 2001. Electronic detection of nucleic acids—a versatile platform for molecular diagnostics. J. Mol. Diag. 3 (2), 74–84.

Wang, J., 2000. Survey and summary. From DNA biosensors to gene chips. Nucleic Acids Res. 28 (16), 3011–3016.

Wang, J., Kawde, A.-N., 2001. Pencil-based renewable biosensor for label-free electrochemical detection of DNA hybridisation. Anal. Chim. Acta 431, 219–224.

Wang, J., Cai, X., Wang, J., Jonsson, C., Palecek, E., 1995. Trace measurements of RNA by potentiometric stripping analysis at carbon paste electrodes. Anal. Chem. 67, 4065–4070.

Wang, J., Cai, X., Rivas, G., Shiraishi, H., Farias, P.A.M., Dontha, N., 1996a. DNA electrochemical biosensor for the detection of short DNA sequences related to the human immunodeficiency virus. Anal. Chem. 68, 2629–2634.

Wang, J., Palecek, E., Nielsen, P.E., Rivas, G., Cai, X., Shiraishi, H., Dontha, N., Luo, D., Farias, P.A.M., 1996b. Peptide nucleic acid probes for sequence specific DNA biosensors. J. Am. Chem. Soc. 118, 7667–7670.

Wang, J., Cai, X., Tian, B., Shiraishi, H., 1996c. Microfabricated thick-film electrochemical sensor for nucleic acid determination. Analyst 121, 965–970.

Wang, J., Rivas, G., Cai, X., 1997a. Screen-printed electrochemical hybridisation biosensor for the detection of DNA sequences from the *Escherichia coli* pathogen. Electroanalysis 9 (5), 395–398.

Wang, J., Rivas, G., Parrado, C., Cai, X., Flair, M.N., 1997b. Electrochemical biosensor for detecting DNA sequences from the pathogenic protozoan *Cryptosporidium parvum*. Talanta 44, 2003–2010.

Wang, J., Cai, X., Rivas, G., Shiraishi, H., Dontha, N., 1997c. Nucleic-acid immobilisation, recognition and detection at chonopotentiometric DNA chips. Biosens. Bioelectron. 12 (7), 587–599.

Wang, J., Rivas, G., Fernandes, J.R., Paz, J.L.L., Jiang, M., Waymire, R., 1998a. Indicator-free electrochemical DNA hybridisation biosensor. Anal. Chim. Acta 375, 197–203.

Wang, J., Fernandes, J.R., Kubota, L.T., 1998b. Polishable and renewable DNA hybridisation biosensors. Anal. Chem. 70, 3699–3702.

Wang, J., Jiang, M., Fortes, A., Mukherjee, B., 1999. New label-free DNA recognition based on doping nucleic-acid probes within conducting polymer films. Anal. Chim. Acta 402, 7–12.

Wang, J., Kawde, A.-N., Sahlin, E., 2000. Renewable pencil electrodes for highly sensitive stripping potentiometric measurements of DNA and RNA. Analyst 125, 5–7.

Wang, J., Kawde, A.-N., Erdem, A., Salazar, M., 2001a. Magnetic bead-based label-free electrochemical detection of DNA hybridisation. Analyst 126, 2020–2024.

Wang, J., Polskj, R., Xu, D., 2001b. Silver-enhanced colloidal gold electrochemical stripping detection of DNA hybridisation. Langmuir 17, 5739–5741.

Wang, J., Xu, D., Erdem, A., Polsky, R., Salazar, M.A., 2002. Genomagnetic electrochemical assays of DNA hybridisation. Talanta 56 (5), 931–938.

Wink, Th., van Zuilen, S.J., Bult, A., van Bennkom, W.P., 1997. Self-assembled monolayers for biosensors. Analyst 122, 43R–50R.

Wojciechowski, M., Sundseth, R., Moreno, M., Henkens, R., 1999. Multichannel electrochemical detection system for quantitative monitoring of PCR amplification. Clin. Chem. 45 (9), 1690–1693.

Xu, D.-K., Ma, L.-R., Liu, Y.-Q., Jiang, Z.-H., Liu, Z.-H., 1999. Development of chemiluminescent biosensing of nucleic acids based on oligonucleotide-immobilised gold surfaces. Analyst 124, 533–536.

Xu, D.-K., Huang, K., Liu, Z., Liu, Y., Ma, L., 2001. Microfabricated disposable DNA sensors based on enzymatic amplification electrochemical detection. Electroanalysis 13 (10), 882–887.

Yang, M., McGovern, M.E., Thompson, M., 1997. Genosensor technology and the detection of interfacial nucleic acid chemistry. Anal. Chim. Acta 346, 259–275.

Yang, M., Yau, H.C.M., Chan, H.L., 1998. Adsorption kinetics and ligand-binding properties of thiol-modified double-stranded DNA on a gold surface. Langmuir 14, 6121–6129.

Yu, C.J., Wan, Y., Yowanto, H., Li, J., Tao, C., James, M.D., Tan, C.L., Blackburn, G.F., Maede, T.J., 2001. Electronic detection of single-base mismatches in DNA with ferrocene-modified probes. J. Am. Chem. Soc. 123, 11155–11161.

Zhao, Y.-D., Pang, D.-W., Hu, S., Wang, Z.-L., Cheng, J.-K., Dai, H.-P., 1999. DNA-modified electrodes. 4. Optimisation of covalent immobilisation of DNA on self-assembled monolayers. Talanta 49, 751–756.